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Survival of Airborne MS2 Bacteriophage Generated from Human Saliva, Artificial Saliva, and Cell Culture Medium

Zhili Zuo,[a] Thomas H. Kuehn,[a] Aschalew Z. Bekele,[b] Sunil K. Mor,[b] Harsha Verma,[b] Sagar M. Goyal,[b] Peter C. Raynor,[c] David Y. H. Pui[a]

Department of Mechanical Engineering, College of Science and Engineering, University of Minnesota, Minneapolis, Minnesota, USA[a]; Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, Saint Paul, Minnesota, USA[b]; Division of Environmental Health Sciences, School of Public Health, University of Minnesota, Minneapolis, Minnesota, USA[c]

Laboratory studies of virus aerosols have been criticized for generating airborne viruses from artificial nebulizer suspensions (e.g., cell culture media), which do not mimic the natural release of viruses (e.g., from human saliva). The objectives of this study were to determine the effect of human saliva on the infectivity and survival of airborne virus and to compare it with those of artificial saliva and cell culture medium. A stock of MS2 bacteriophage was diluted in one of three nebulizer suspensions, aerosolized, size selected (100 to 450 nm) using a differential mobility analyzer, and collected onto gelatin filters. Uranine was used as a particle tracer. The resulting particle size distribution was measured using a scanning mobility particle sizer. The amounts of infectious virus, total virus, and fluorescence in the collected samples were determined by infectivity assays, quantitative reverse transcription-PCR (RT-PCR), and spectrofluorometry, respectively. For all nebulizer suspensions, the virus content generally followed a particle volume distribution rather than a number distribution. The survival of airborne MS2 was independent of particle size but was strongly affected by the type of nebulizer suspension. Human saliva was found to be much less protective than cell culture medium (i.e., 3% tryptic soy broth) and artificial saliva. These results indicate the need for caution when extrapolating laboratory results, which often use artificial nebulizer suspensions. To better assess the risk of airborne transmission of viral diseases in real-life situations, the use of natural suspensions such as saliva or respiratory mucus is recommended.

The potential involvement of virus aerosols in the transmission of human respiratory diseases, although still under considerable debate, has led to increased public concern. Several studies have found that a variety of respiratory viruses, including influenza virus and severe acute respiratory syndrome (SARS) coronavirus, could be present at high concentrations in human saliva and respiratory mucus (1–3). When infected individuals cough, sneeze, speak, or simply breathe, particles of saliva and/or respiratory mucus that carry viruses can be easily generated (4, 5), resulting in an increased risk of viral infection by aerosols.

In an effort to understand and control transmission of viral diseases via aerosols, researchers have generated airborne viruses in laboratories to study their infectivity and survival (i.e., the ability to remain infectious) since the 1930s. Laboratory-generated virus aerosols are commonly produced from liquid suspensions using pneumatic nebulizers such as Collison nebulizers, as the wet-dispersion technique simulates many dispersion processes of viruses in the natural environment (6). However, the composition of liquid suspensions from which virus aerosols are generated (also known as nebulizer suspensions) is known to affect the infectivity/survival of airborne viruses (7, 8). Given that many laboratory studies use artificial nebulizer suspensions (e.g., cell culture media) that do not mimic natural release of virus aerosols from body secretions (e.g., human saliva), it has been suggested that survival of airborne viruses determined in laboratories may not represent that in real-life situations (9).

To better simulate the generation of virus aerosols from human saliva, several researchers have developed a recipe for making artificial saliva and have used it as a nebulizer suspension (10–13). The same artificial saliva was later adopted by ASTM to evaluate the decontamination efficacy of air-permeable materials and surfaces challenged with bioaerosols (14, 15). MS2 bacteriophage aerosolized from artificial saliva has been found to survive better than that from deionized (DI) water but no better than that from 0.25% tryptone solution or 0.3% beef extract (10, 13), suggesting that artificial saliva may indeed affect the survival of airborne viruses differently from other commonly used artificial nebulizer suspensions. However, it remains unclear how closely artificial saliva could represent human saliva in terms of preserving airborne virus infectivity.

The literature on the effect of human saliva on airborne viruses and comparison with other nebulizer suspensions is limited. In one study (16), survival of airborne bacteriophage T3 from saliva and 0.1% peptone was found to be similar at relative humidities (RH) ranging from 20% to 80% but lower than that from 0.1 M NaCl at low to middle RH. At high RH, the highest survival was observed using saliva, followed by peptone and salt. In another study (17), saliva was found to offer less protection against airborne encephalomyocarditis virus than Hanks balanced salt solution over a wide range of RH, and the virus decay in saliva was even larger than that in water at below 40% RH. These results indicate that saliva may not necessarily be more effective in maintaining airborne virus infectivity than artificial nebulizer suspensions, de-

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Address correspondence to Zhili Zuo, zuoxx011@umn.edu.
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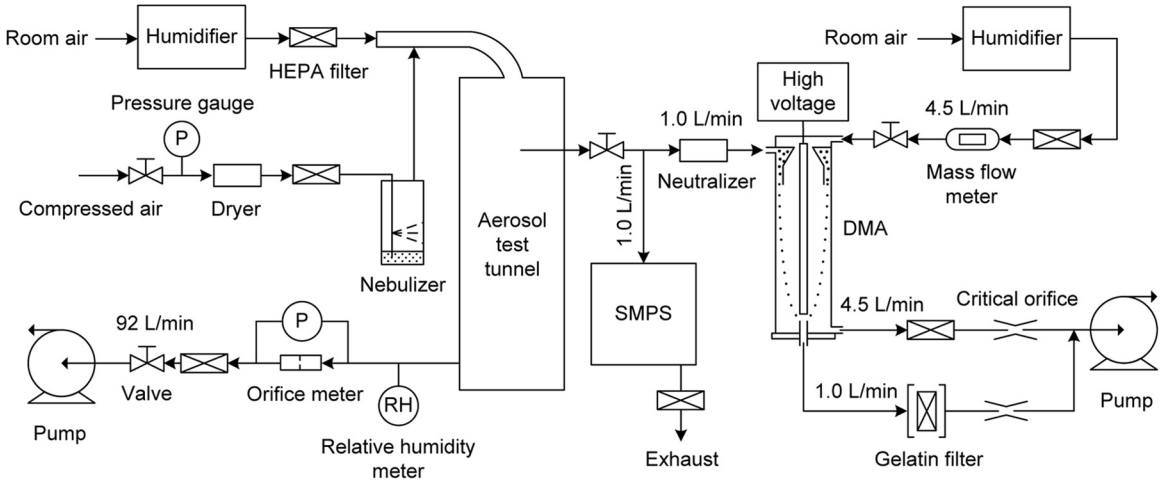

FIG 1 Schematic diagram of the experimental setup for the measurement of virus aerosol.

pending on RH. However, in both studies, spray guns were used to produce micrometer-size particles, and the virus aerosols were collected by liquid impingers. One limitation of liquid impingers is that they can provide only particle size-integrated results. As demonstrated previously, particle size can significantly affect survival of airborne viruses (18) as well as their removal by filtration (19). Therefore, it would be interesting to explore how human saliva affects virus in aerosols as a function of particle size. In addition, liquid impingers are inefficient in collecting submicrometer-size particles (20). Consequently, the reported infectivity and survival results represent mainly those for micrometer-size particles. What happens to virus aerosol particles of <0.5 μm, the size of most respiratory particles (21, 22), remains unclear.

The objective of this study was to determine how human saliva could affect the infectivity and survival of airborne MS2 bacteriophage in the submicrometer size range and to compare the use of human saliva with that of artificial saliva and cell culture medium. Due to its nonpathogenicity and ease of propagation, MS2 is one of the most popular surrogates for human-pathogenic viruses and has been extensively used in various virus aerosol studies, including investigation of aerosol generation techniques (23), sampler evaluation (20, 24), virus survival (10, 18), virus inactivation (12, 25), and virus filtration (26). For these reasons, MS2 was selected in this study.

MATERIALS AND METHODS

Virus stock. Bacteriophage MS2 is a small (27-nm), tailless, nonenveloped, single-stranded RNA coliphage. MS2 bacteriophage (ATCC 15597-B1) was propagated and titrated using *Escherichia coli* famp (ATCC 700891) as host cells. Briefly, 1 ml of virus stock was mixed with 100 ml of log-phase *E. coli* grown in 3% (wt/vol) tryptic soy broth (TSB). After incubation at 37°C for 20 h with shaking at 60 rpm, the culture was centrifuged at 6,000 × *g* for 15 min and the supernatant was filtered through a 0.45-μm cellulose acetate filter (Vanguard International, Neptune, NJ). The resulting virus stock was aliquoted in 2-ml vials and stored at −80°C until used.

Nebulizer suspensions. Three types of nebulizer suspensions were evaluated: human saliva (HS), artificial saliva (AS), and artificial saliva with no mucin (ASNM). ASNM was tested to further evaluate the effect of mucin on the survival of airborne MS2. (i) Whole human saliva was collected from a subject under unstimulated conditions, at least 2 h after eating and drinking, using the spitting method (27). The subject first rinsed the mouth thoroughly using DI water and sat upright with the head slightly tilted forward and the eyes open. Saliva was then allowed to accumulate in the mouth, and the subject spit it out every 1 min into a 50-ml tube until ~45 ml of saliva was collected. The collected saliva was treated with 455 μg/ml streptomycin and 1.5 μg/ml amphotericin B (Fungizone) to inhibit microbial growth. Since the composition of saliva varies on a daily basis (28) and is also affected by freezing and thawing (e.g., with formation of white precipitates) (29), saliva collected on different days was pooled, well mixed, aliquoted into 50-ml tubes, and stored at 4°C until used (within 7 days of collection). (ii) Artificial saliva was prepared using the same recipe as described elsewhere (11). It consisted of ~0.3% various salts and 0.3% mucin from porcine stomach (M1778; Sigma Chemical Co., St. Louis, MO) to simulate the electrolytes and mucus in human saliva (HS), respectively. Mucin-free artificial saliva was prepared using the salts only. On each day of testing, 4.5 ml of thawed MS2 stock was diluted in 40.5 ml of one the three nebulizer suspensions supplemented with 2 ml of uranine (0.625 g/ml) (Fluka, Buchs, Switzerland) and 0.1 ml of antifoam Y-204 (Sigma). Uranine was used as a fluorescent particle tracer (18, 24, 30), which allowed the quantitation of particle transport loss in the test system. The titer of freshly prepared nebulizer suspensions ranged from 2×10^8 to 6×10^8 PFU/ml.

Experimental setup and test procedure. The experimental setup schematically shown in Fig. 1 has been described elsewhere (18). The main element is a one-pass vertical aerosol test tunnel, which has been used in different virus aerosol studies (18, 19, 24, 31, 32). Before each experiment, the tunnel was first purged using HEPA-filtered air at 92 liters/min for 15 min to remove any residual particles. MS2 bacteriophage was then aerosolized from one of the nebulizer suspensions using a six-jet Collison nebulizer (model CN25; BGI Inc., Waltham, MA) operated at 10 lb/in^2 gauge. The generated MS2 aerosol was mixed and diluted with humidity-controlled and HEPA-filtered room air, entering the tunnel at a flow rate of 92 liters/min. The virus aerosol was sampled with a scanning mobility particle sizer (SMPS) (model 3034; TSI, Inc., Shoreview, MN) at 1 liter/min to measure the particle number distribution from 10 nm to 470 nm. Meanwhile, the aerosol was charge equilibrated to the Boltzmann distribution using a polonium-210 neutralizer. Particles with size of 100, 200, 300, 400, and 450 nm were selected with a differential mobility analyzer (DMA) (model 3071; TSI), one size at a time. The size-classified virus aerosol particles were then collected with a 25-mm-diameter gelatin filter (SKC, Inc., Eighty Four, PA) held in a stainless steel holder (Millipore Corp., Bedford, MA) at 1.0 liter/min for 15 min. Gelatin filters have a very high collection efficiency for MS2 aerosol (33). The sampling time was limited to 15 min in order to minimize desiccation, which adversely affects the infectivity of the collected virus. Immediately after sampling, the

gelatin filter was broken apart using a sterile forceps and dissolved in 1 ml of 1.5% beef extract–0.05 M glycine solution (pH 7.2), followed by vortexing (American Scientific Products, McGaw Park, IL) at maximum speed for ~10 s six times at 1-min intervals. Any unsampled virus aerosol was removed with a HEPA filter located at the outlet of the tunnel.

The experiments were performed at an RH of 45% ± 5% and a temperature of 22 to 24°C with three replicates for each nebulizer suspension. All equipment, including the nebulizer, filter holders, and forceps, was sterilized prior to testing. The entire setup was enclosed by secondary containment with exhaust ventilation to prevent the release of aerosols into the surrounding environment.

Sample analysis. Before and after each experiment, a 1-ml sample of nebulizer suspension was collected. Each nebulizer suspension sample and gelatin filter sample was split into three portions. The first portion was diluted in 0.01-mol/liter NaOH, and the concentration of fluorescence was measured with a spectrofluorometer (model RF-5201PC; Shimadzu Scientific Instruments, Columbia, MD) at excitation and emission wavelengths of 485 nm and 515 nm, respectively. The remaining two portions were stored at −80°C until used in analysis of infectious virus (IV) and total virus (TV).

Infectious virus was enumerated using a double-agar-layer plaque assay (34). Briefly, serially diluted samples in phosphate-buffered saline were added to 4 ml of 0.75% tryptic soy agar (TSA) maintained at 48°C along with 0.1 ml of log-phase *E. coli*. This "top agar" was poured onto preprepared 1.5% TSA "bottom agar" plates and allowed to solidify. The plates were then inverted, incubated at 37°C for 18 h, and examined for the production of viral plaques. The plaques were counted, and viral titers were expressed as PFU/ml.

Total virus was quantified by quantitative reverse transcription-PCR (qRT-PCR), as described elsewhere (18). Since qRT-PCR measures both infectious and noninfectious virus, the results are referred to as for total virus. Briefly, viral RNA was extracted from 140 μl of each sample and eluted in 40 μl of elution buffer using the QIAamp viral RNA kit (Qiagen, Valencia, CA). The extracted viral RNA (3 μl) was mixed with specific primers and probe (35) (one-step RT-PCR kit; Qiagen) to a final volume of 20 μl. qRT-PCR was then performed in duplicate in a Mastercycler ep Realplex2 thermocycler (Eppendorf, Hamburg, Germany). Viral RNA extracted from the virus stock with a known titer was serially diluted in RNase-free water and used to construct standard curves, which translated threshold cycle (C_T) values into projected titers in PFU/ml. Similar standard curves were obtained by serially diluting MS2 stock in 3% TSB, HS, and AS, followed by RNA extraction, which suggested little RNA degradation due to different nebulizer suspensions.

Data analysis. To evaluate the effect of nebulization on the stability of virus infectivity and viral RNA in the nebulizer suspensions, two parameters, γ_{IV} and γ_{TV}, were calculated: $\gamma_{IV} = (C_{IV,neb}/C_{F,neb})_a/(C_{IV,neb}/C_{F,neb})_b$ and $\gamma_{TV} = (C_{TV,neb}/C_{F,neb})_a/(C_{TV,neb}/C_{F,neb})_b$. These parameters compare the concentration ratio of infectious ($C_{IV,neb}$) or total ($C_{TV,neb}$) virus to fluorescence intensity ($C_{F,neb}$) in the nebulizer suspension before (*b*) and after (*a*) nebulization. The inclusion of $C_{F,neb}$ in the equations takes into account the possible artificial increase of the virus concentration due to water evaporation from the suspensions during nebulization (18, 24).

To understand how viral content (either infectious or total virus) was distributed among various particle sizes, we defined the virus size distribution, $dC_V(D_p)/d\log_{10}D_p$, a size distribution function particularly for particles carrying virus (18, 20): $dC_V(D_p)/d\log_{10}D_p \approx [C_{V,gel}(D_p)V_{gel}/Q_{gel}t(\Delta\log_{10}D_p)] \times [1/f_{+1}(D_p)P(D_p)]$, where $C_{V,gel}$ is the concentration of infectious virus or total virus recovered from the gelatin filter at a certain particle size D_p, V_{gel} is the volume of gelatin filter sample, Q_{gel} and t are the gelatin filter sampling flow rate and sampling time, $\Delta\log_{10}D_p$ is the logarithm of the width of the size interval of the DMA, f_{+1} is the fraction of singly positively charged particles (36), and P is the penetration of particles through the DMA (37).

The amount of infectious or total virus carried per particle, $\nu(D_p)$, was calculated as the ratio of the total amount of virus collected by the gelatin filter to the total number of particles measured by the SMPS at a given particle size: $\nu(D_p) \approx [C_{V,gel}(D_p)V_{gel}/Q_{gel}t\Delta C_{n,SMPS}(D_p)] \times [1/f_{+1}(D_p)P(D_p)]$, where $\Delta C_{n,SMPS}$ is the particle number concentration measured by the SMPS within a size interval with geometric mean diameter D_p.

To quantify how efficiently infectious virus was recovered, the relative recovery of infectious virus (RR_{IV}) was calculated: $RR_{IV}(D_p) = [C_{IV,gel}(D_p)/C_{F,gel}(D_p)]/(C_{IV,neb}/C_{F,neb})$, where $C_{IV,neb}$ and $C_{F,neb}$ are the averaged values before and after nebulization. The infectious virus concentrations are normalized by fluorescence concentrations to take into account any artificial loss of virus infectivity due to the transport loss of particles (e.g., sedimentation and deposition) in the test system. If there is no inactivation of virus (i.e., 100% survival), then $RR_{IV} = 1$, assuming no measurement error. Therefore, RR_{IV} represents the fraction of infectious virus recovered relative to the fluorescence recovered, and it serves as an indication of the survival of airborne virus (18, 24). Similarly, we also calculated relative recovery of total virus (RR_{TV}): $RR_{TV}(D_p) = [C_{TV,gel}(D_p)/C_{F,gel}(D_p)]/(C_{TV,neb}/C_{F,neb})$.

One method to quantify the survival of airborne virus is to compare the ratio of infectious virus to total (both infectious and noninfectious) virus (10, 13, 18, 38, 39). The lower the ratio, the poorer the survival is. Here, the infectious-to-total virus ratio (ITR) in the aerosol sample was normalized by the same ratio in the nebulizer suspensions: $ITR(D_p) = [C_{IV,gel}(D_p)/C_{TV,gel}(D_p)]/(C_{IV,neb}/C_{TV,neb})$, where $C_{IV,neb}$ and $C_{TV,neb}$ are the averaged values before and after nebulization.

Data obtained for different nebulizer suspensions at different particle sizes were statistically analyzed using one-way or multiway analysis of variance (ANOVA) in MATLAB R2010b. A P value of ≤0.05 was considered statistically significant.

TABLE 1 Geometric means of γ_{IV} and γ_{TV} for the four nebulizer suspensions

Nebulizer suspension	Geometric mean (95% confidence interval)	
	γ_{IV}	γ_{TV}
Cell culture medium (3% TSB)[a]	1.07 (0.44, 2.62)	0.96 (0.48, 1.93)
Human saliva	0.86 (0.63, 1.18)	0.87 (0.41, 1.87)
Artificial saliva	1.08 (0.95, 1.21)	0.66 (0.36, 1.19)
Artificial saliva no mucin	1.13 (0.79, 1.61)	1.12 (0.90, 1.41)

[a] Data are from reference 18.

RESULTS

In a previous study (18), we measured MS2 bacteriophage aerosolized from a cell culture medium (i.e., 3% TSB) supplemented with the same amounts of uranine and antifoam as in this study. Therefore, those results are also presented here for comparison purposes.

Stability of virus in nebulizer suspensions. Table 1 shows the geometric means and 95% confidence interval of γ_{IV} and γ_{TV} for the four nebulizer suspensions. Neither γ_{IV} nor γ_{TV} was significantly different from unity, suggesting that the composition of the nebulizer suspension, along with uranine and antifoam, did not inactivate the virus. In addition, one-way ANOVA showed that there was no statistically significantly difference in γ_{IV} ($P = 0.429$) or γ_{TV} ($P = 0.142$) among the four nebulizer suspensions.

Particle size distributions. Typical SMPS-measured particle number distributions of virus aerosols generated from the four nebulizer suspensions and their particle statistics are shown in Fig. S1 in the supplemental material. The size distributions of TSB and ASNM were log-normal with count median diameters of 76.5 and 64.3 nm, respectively. However, the sizes of particles generated

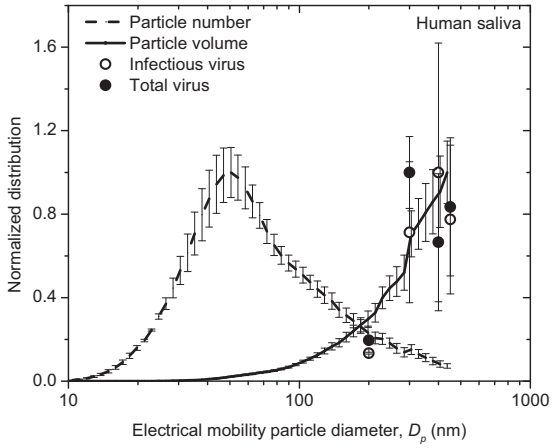

FIG 2 Normalized particle number, particle volume, infectious virus, and total virus size distributions for human saliva aerosol. Virus size distributions and particle size distributions were normalized by their highest values and superimposed for easy comparison. Values are means ± one standard deviation ($n = 3$). Data points for infectious virus and total virus size distributions at 100 nm were not plotted since no virus was recovered. Similar plots for cell culture medium (i.e., 3% TSB), artificial saliva, and artificial saliva without mucin are available in Fig. S2 in the supplemental material.

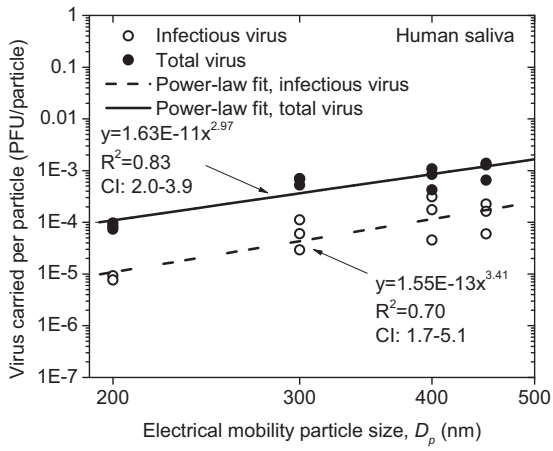

FIG 3 Amounts of infectious and total virus carried per particle as a function of particle size for human saliva aerosol. Also shown are the curve fitting results with r^2 values and 95% confident intervals (CI) of the slopes, where x represents particle size in nm and y represents virus carried per particle in PFU/particle. Data points at 100 nm were not plotted since no virus was recovered. Similar plots for cell culture medium (i.e., 3% TSB), artificial saliva, and artificial saliva without mucin are available in Fig. S3 in the supplemental material.

from HS were bimodally distributed. The primary mode at ~50 nm had a magnitude almost twice that of the secondary mode at ~80 nm. Unlike the three nebulizer suspensions mentioned above, the particle size distributions of AS measured before, during, and after the experiment were multimodal and all different (see Fig. S1 in the supplemental material). During a nebulization period of ~90 min, the two peaks at 15 and 33 nm continued to diminish (with decreases in number concentration of 62% and 25%, respectively), while a third peak at ~110 nm gradually appeared (with an increase in number concentration of 53%). This large variation with time indicates an unstable output of the Collison nebulizer when AS was nebulized.

Virus size distributions. Figure 2 and Fig. S2 in the supplemental material present the infectious virus and total virus size distributions for the four nebulizer suspensions. The concentration of airborne virus generally increased with particle size, and the maximum mean concentrations of infectious virus (at 400 or 450 nm) were 3,600, 25, 220, and 33 PFU/cm^3 for TSB, HS, AS, and ASNM, respectively, which were several orders of magnitude lower than particle number concentrations (see Fig. S1 in the supplemental material). For all four nebulizer suspensions, the infectious virus and total virus size distributions followed the particle volume distribution better than the particle number distribution, despite the large error bars.

Virus carried per particle. As shown in Fig. 3 and Fig. S3 in the supplemental material, the amounts of infectious virus (IV) and total virus (TV) carried per particle increased with particle size for each of the four nebulizer suspensions. The results of curve fitting suggest that the association of IV and TV with particle size reasonably followed a power law, where the power was not significantly different from 3. However, even for the largest particles (450 nm), the amounts of IV and TV were much lower than 1 PFU/particle.

Relative recovery of infectious virus. RR_{IV} is plotted in Fig. 4 as a function of particle size for the four nebulizer suspensions. Two-way ANOVA showed that RR_{IV} was independent of particle size ($P = 0.168$) but significantly depended on the type of nebulizer suspension ($P < 0.001$). TSB and AS had similar RR_{IV}s, much higher than those of HS and ASNM. Particle size-averaged RR_{IV}s were 0.285, 0.032, 0.218, and 0.024 for TSB, HS, AS, and ASNM, respectively.

Relative recovery of total virus. Similar to the case for RR_{IV}, RR_{TV} (Fig. 5) was also independent of particle size (two-way ANOVA, $P = 0.853$). Particle size-averaged RR_{TV}s were 0.265, 0.130, 0.414, and 0.215 for TSB, HS, AS, and ASNM, respectively. Therefore, RR_{TV}s were generally comparable for the four nebulizer suspensions, as confirmed by statistical analysis (two-way ANOVA, $P = 0.053$).

Infectious-to-total virus ratio. Figure 6 shows the ITRs for the four nebulizer suspensions at different particle sizes. Similar to the case for RR_{IV} and RR_{TV}, particle size had little effect on ITR (two-

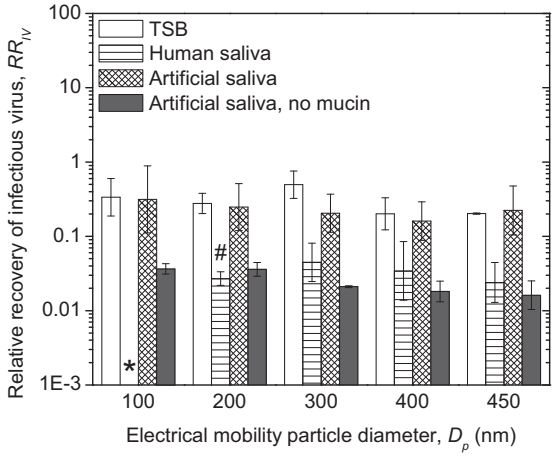

FIG 4 Relative recovery of infectious virus. Each bar represents the geometric mean ± one standard deviation of the mean ($n = 3$). *, infectious virus was below the limit of detection in all three samples. #, infectious virus was recovered in only two of the three samples. Results for TSB are from reference 18.

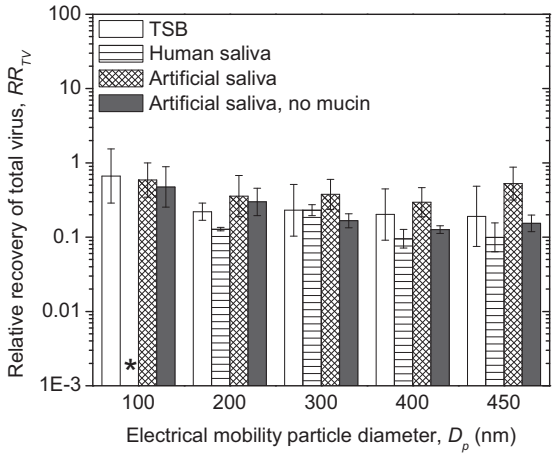

FIG 5 Relative recovery of total virus. Each bar represents the geometric mean ± one standard deviation of the mean ($n = 3$). *, viral RNA was below the limit of detection in all the three samples. Results for TSB are from reference 18.

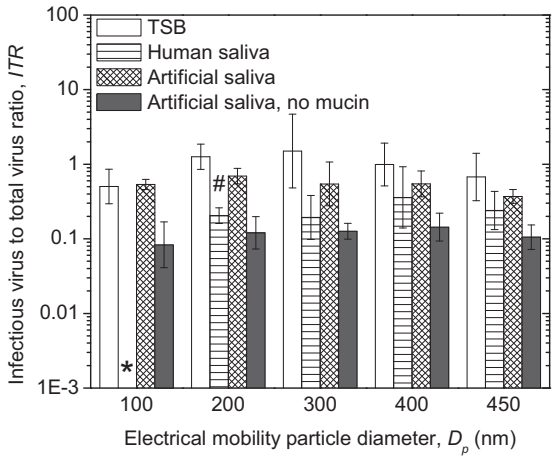

FIG 6 Infectious-to-total virus ratio. Each bar represents the geometric mean ± one standard deviation of the mean ($n = 3$). *, infectious virus and viral RNA were below the limit of detection in all three samples. #, infectious virus was recovered in only two of the three samples. Results for TSB are from reference 18.

way ANOVA, $P = 0.399$). However, ITR was strongly affected by the type of nebulizer suspension (two-way ANOVA, $P < 0.001$). TSB gave the highest particle size averaged ITR (0.915), followed by AS (0.527), HS (0.242), and ASNM (0.114).

DISCUSSION

Effect of nebulization and sampling on virus survival. The high shear stress during nebulization often substantially reduces the viability of bacteria (40). However, values of γ_{IV} and γ_{TV} (Table 1) indicate that neither the infectivity nor the viral RNA of MS2 bacteriophage was affected by the nebulization stress. This finding is consistent with the literature, where both enveloped and nonenveloped viruses were aerosolized from cell culture media (18, 24, 30, 31). The four nebulizer suspensions gave similar γ values, suggesting that the insensitivity of virus to nebulization stress is not unique for cell culture media but occurs for other suspensions also. One possible reason for the insensitivity could be the small physical size and inertia of the virus, which makes it experience lower nebulization stress than bacteria (31).

Filters are usually not recommended for collecting infectious virus aerosols because of the detrimental effect of desiccation during sampling (41). To evaluate whether gelatin filters would impose sampling stress on the collected virus, an additional experiment was designed and repeated in triplicate by running two gelatin filters in parallel. After sampling airborne MS2 for 3 min, one filter was immediately analyzed by plaque assay, while the other was exposed to HEPA-filtered air for 15 min (to simulate the level of desiccation experienced by the virus) and then analyzed. One-way ANOVA suggested that there was no significant difference ($P = 0.737$) between exposed ($7.4 \pm 1.1 \times 10^5$ PFU/ml) and unexposed ($7.7 \pm 0.9 \times 10^5$ PFU/ml) samples. These results are similar to what has been reported elsewhere (25) and further confirm that gelatin filters caused minimum sampling stress, which may be attributed to the high moisture content of the gelatin.

As there was little nebulization and sampling stress, the loss of virus infectivity in the present experiments seems to occur primarily in the aerosol phase.

Particle size distribution. The particle size distributions generated from the four nebulizer suspensions did not reflect the physical size of the virus itself (see Fig. S1 in the supplemental material). Instead, they were determined primarily by the compositions of the nebulizer suspensions. Due to the higher volume fraction of solid material (solute) in the nebulizer suspension, the size distribution of TSB gave a mean diameter larger than that of ASNM. Interestingly, the size distribution of HS was bimodal, indicating two different sources of particles. One source was certainly the various chemicals (e.g., salts and uranine) dissolved in the suspension, similar to the case for TSB and ASNM. The other source might come from salivary micelles. As revealed by electron microscopy, salivary micelles are multicomponent protein complexes in HS with globular structures (42). They are often in the form of individual particles or aggregates, ranging from 50 to 400 nm (42, 43), with mean sizes close to the second mode of the bimodal distribution. The addition of mucin to ASNM changed the size distribution from log-normal to multimodal (see Fig. S1 in the supplemental material). Mucins are large glycoproteins with long-chain structures that tend to be entangled with each other (44). Due to the high recirculation rate of the suspension during nebulization (e.g., once every 6 s) (45), shear stress might gradually break up these entanglements, thus causing unstable output from the nebulizer.

Virus size distribution and amount of virus carried per particle. Similar to TSB (18), HS, AS, and ASNM produced infectious and total virus size distributions that were generally represented by the particle volume distribution (Fig. 2; see Fig. S2 in the supplemental material), suggesting that the amount of virus carried per particle was proportional to particle volume. This finding is further supported by the curve fitting results for IV and TV (Fig. 3; see Fig. S3 in the supplemental material), which showed a power of ~3. A similar trend was also observed for various animal viruses aerosolized from cell culture media in the submicrometer (18) and micrometer size ranges (24, 30). Assuming that one PFU represents one infectious virus, values much lower than 1 PFU/particle for IV and TV (Fig. 3; see Fig. S3 in the supplemental material) indicate that only a small fraction of the generated particles

actually carried virus. This is because conventional Collison nebulizers inevitably generate many virus-free residual particles, even if suspensions with high virus titers are used, as explained previously (18). Therefore, compared with cell culture medium, the use of non-cell culture medium did not change how virus was distributed among or carried by particles of different sizes.

Effect of nebulizer suspensions on virus survival. Virus survival was a strong function of the type of nebulizer suspension. Survival of airborne MS2 was highest for TSB and AS, moderate for HS, and lowest for ASNM, as indicated by the values of RR_{IV} (Fig. 4) and ITR (Fig. 6). Although it is well known that the composition of a nebulizer suspension plays a significant role in survival of airborne viruses (7, 8), how it preserves or reduces virus infectivity is less understood.

Multiple inactivation mechanisms have been proposed and reviewed regarding the effect of nebulizer suspension composition (46, 47). However, for MS2 bacteriophage, the loss of its survival (RR_{IV} of <1 and ITR of <1) could be most plausibly explained by the exposure to an air-water interface (AWI), where the virus experiences "deforming forces," which causes irreversible folding and rearrangement of virus protein molecules and thus inactivates the virus (48, 49). Considering the moderate RH used during the experiments and the highly hygroscopic nature of uranine (50) and other salts (e.g., NaCl) used in the nebulizer suspensions, the generated particles might carry a substantial water content rather than being completely dried, thus creating an AWI. MS2 bacteriophage, though nonenveloped, is very hydrophobic (51) and thus tends to accumulate at the AWI. The increased concentration of salts in the generated droplets due to water evaporation decreases the size of the virus double layer, further promoting virus adsorption at the AWI (49, 51). All these observations suggest that inactivation of MS2 in the aerosol phase could be a combined result of exposure to AWI and the hygroscopic nature of the particles.

The addition of proteins to liquid suspensions may reduce the solution surface tension, which makes it more difficult for the virus to reach the AWI, thereby reducing virus inactivation (49, 51). This may be the reason why MS2 nebulized from protein-rich TSB survived much better than that from salt-dominant ASNM. Other proteinaceous solutions, such as peptone, were also reported to increase the survival of airborne MS2 (48, 52).

The use of mucin significantly enhanced virus survival (Fig. 4 and 6). Woo et al. (12) showed that the cross-linking network of mucin forms a thin layer to encapsulate virus in aerosols. This layer may reduce virus exposure to AWI and thus boost survival. However, the protection level offered by mucin may not monotonically increase with its concentration. For example, Schoenbaum et al. (53) recovered much less infectious pseudorabies virus when the mucin concentration was increased from 1% to 2%. HS, although it also contains mucin, gave a much lower survival than AS, suggesting that exposure to AWI was probably not the only inactivation mechanism. Airborne foot-and-mouth disease virus has been found to be particularly sensitive to an undefined organic molecule present in bovine salivary fluid (54). A similar situation may also exist for HS and MS2, especially considering the numerous trace components in HS (28) and that many of them possess antiviral activities (55).

Instability of viral RNA in aerosol. The RR_{TV} of <1 (Fig. 5) suggests that viral RNA was not fully preserved in the aerosol. The average values for $(1 - RR_{TV})/(1 - RR_{IV})$ (i.e., the fraction of virus inactivation due to viral RNA damage) were 0.97, 0.89, 0.72, and 0.76 for TSB, HS, AS, and ASNM, respectively, suggesting that inactivation of airborne MS2 was largely due to its damaged viral RNA. Inactivation of several enveloped viruses, however, has been shown to result mainly from their damaged viral capsid and/or envelope proteins (18). Perhaps a virus envelope could protect viral RNA more effectively than viral capsid in aerosol. The exact inactivation mechanisms potentially could be determined using the promising methods described by Wigginton et al. (56).

The instability of viral RNA also raises the question of using viral nucleic acids as a particle tracer for virus aerosol studies. If viral nucleic acids degrade in aerosol, then the infectious-to-total virus ratio (ITR), which has often been used (10, 13, 18, 38, 39) as an indicator for airborne virus survival, may underestimate it. An ideal particle tracer must be highly stable in aerosol and easily quantifiable so that the tracer concentration is directly proportional to the virus concentration. From this viewpoint, uranine is still the most reliable particle tracer (30, 41).

Effect of particle size on survival. Airborne MS2 survival was a weak function of particle size (Fig. 4 and 6). However, large particle size has been found to increase virus survival due to the shielding effect (12, 18) of other material. It is possible that the size of particles sampled (100 to 450 nm) was already much larger than the physical size of MS2 (27 nm), so a further increase in particle size did not enhance survival. Woo (13) extended the measurement of MS2 down to 30 to 230 nm and did observe an increase of survival with increased particle size.

Use of natural nebulizer suspensions for risk assessment. To assess the risk of airborne transmission of viral diseases, information on the survival of viruses in aerosol is of critical importance. However, as clearly demonstrated in this study, artificial nebulizer suspensions, even artificial saliva, did not produce the same effect as human saliva on the infectivity and survival of airborne MS2 bacteriophage. A significant difference in the survival of several veterinary viruses between cell culture media and animal salivary fluid was also reported (54, 57–59). These findings strongly suggest that the use of artificial nebulizer suspensions may over- or underestimate the survival of airborne viruses in real-life situations and therefore negatively impact risk assessment. To increase the clinical or epidemiological value of a study, the use of natural nebulizer suspensions is recommended. However, we should be cautious about (i) the potential unstable aerosol output from nebulizers (e.g., AS in our study), particularly when aerosol has to be sampled for a prolonged time period, and (ii) the possible PCR inhibition by natural suspensions, as shown elsewhere (60).

Limitations. One of the main limitations is that the saliva used in this study came from only one subject. Given that there exists large intersubject variation in terms of salivary components and antiviral properties of saliva (28, 55, 61), saliva from multiple donors should be tested in the future to determine variation in the population and to allow intrastudy comparison. In addition, MS2 has been found to be a poor surrogate for certain human and animal viruses in the aerosol phase (18, 24), and the effect of nebulizer suspensions generally varies for different viruses (7). Therefore, it is difficult to predict the effect of human saliva and artificial suspensions on airborne human viruses based on the results with MS2. It would be interesting to test better surrogate viruses aerosolized from human saliva. Also, only one value of RH was tested in these experiments. Several studies (54, 57–59) have found that RH affects how cell culture media and natural fluids protect airborne viruses. Moreover, although uranine was used as

a particle tracer, it changed the composition of the nebulizer suspensions and could have potentially affected virus survival. For example, uranine has been shown to be toxic to airborne virus (62). The effect of RH and uranine on the survival of airborne viruses warrants further investigation.

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